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(54) Title: TISSUE SAMPLE PROCESSING

(57) Abstract

Use of a compound of the general formula (I) $R_1$ - C(O)- O - $R_2$ wherein $R_1$ is a straight chain $C_{3-23}$ alkyl group, a straight chain $C_{3-23}$ alkenyl group, a straight chain $C_{5-23}$ alkadienyl group, or a straight chain $C_{7-23}$ alkatrienyl group, where the alkyl, alkenyl, alkadienyl and alkatrienyl group may optionally be substituted with 1 or 2 OH groups; $R_2$ is a straight chain $C_{1-10}$ alkyl group which may optionally be substituted with 1 or 2 OH groups; with the proviso that the total number p of carbon atoms in $R_1$ and $R_2$ is in the range of $12 \leq p \leq 26$; or a mixture of such compounds in the clarification and/or deparaffination procedures in the processing of histological tissue samples. $R_2$ is preferably a $C_{1-6}$ alkyl group, in particular a $C_{1-4}$ alkyl group, and p is preferably in the range $12 \leq p \leq 18$. Specific examples of formula (I) is n-butyl decanoate and n-butyl dodecanoate. In the use according to the invention, the compound (I) may be used in combination with other components such as saturated hydrocarbon solvents, vegetable oils, other esters of mono-, di- or tricarboxyl acids, or terpenes.

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# TISSUE SAMPLE PROCESSING

## FIELD OF THE INVENTION

The present invention relates to the use of certain ester compounds in the processing of tissue samples that are to be subjected to histological examination.

## BACKGROUND OF THE INVENTION

When preparing human or animal tissues for histological studies, e.g. examination for the presence of malignant cells, it is necessary to pass the tissue sample through a number of steps comprising fixation, typically using a fixing agent such as formaldehyde; dewatering, typically using various mixtures of water and a lower alkanol such as ethanol; clarification; paraffin casting of the clarified tissue sample; the cutting of thin sections from the paraffin cast; deparaffination of the thin section; and staining of the section.

The purpose of the clarification step is to provide a transition (in terms of hydrophillic/lipophilic properties) between on the one hand the dehydrating agent (which must typically be miscible with water) and on the other hand the paraffin. Likewise, the purpose of the deparaffination step is to remove the paraffin from the thin section on a microscope glass slide to prepare the sample for staining by means of agents dissolved in alcoholic or aqueous media.

Traditionally, the clarification and deparaffination steps have involved the use of aromatic solvents such as toluene and, in particular, xylene (typically in the form of technical quality xylene consisting of a mixture of o-, m- and p-xylene). However, in spite of the development of automatic tissue preparation machines, the high toxicity of xylene has lead to a need to replace xylene with a less toxic chemical.

Thus, a number of attempts at realizing such a replacement have been made, examples of potential replacement products being hydrocarbons such as odourless kerosene or n-paraffins, terpenes such as d-limonene, lactate compounds and other organic solvents. Thus, d-limonene which is an unsaturated terpene hydrocarbon solvent extracted from citrus fruits has been applied for some years as a replacement for xylene in histological tissue preparation. However, although the product works technically well, the strong citrus odour together with inherent skin irritation problems has severely limited the practical use of this compound.

Xylene replacement products which are to be acceptable from a functional and work environmental point of view must fulfil a number of criteria such as low toxicity, low vapour pressure, weak odour, good thermal stability up to 70°C, low coloration, inert to human and animal tissue, no influence on tissue staining, and good long-term stability at ambient temperature. Also, the replacement products should be environmentally acceptable in that they should be non-toxic to fish and bacteria and also be readily biodegradable.

An attempt has also been made at using an ester between a branched alkanol and an alkanoic acid, namely 2-ethyl-hexyl dodecanoate. However, this compound has proved functionally unsatisfactory in that samples of high-fat tissue gives rise to problems because it becomes difficult to cut thin sections from the paraffin castings due to the tissue becoming fragile and crumbly.

It has now surprisingly been found that by using certain esters of certain straight-chain fatty acids and certain straight-chain alcohols, the above-described disadvantages in xylene and known xylene replacement products can be eliminated or substantially reduced.

SUMMARY OF THE INVENTION

Consequently, the present invention concerns the use of a compound of the general formula I

$$R_1 - C(O) - O - R_2$$

wherein $R_1$ is a straight chain $C_{3-23}$ alkyl group, a straight chain $C_{3-23}$ alkenyl group, a straight chain $C_{5-23}$ alkadienyl group, or a straight chain $C_{7-23}$ alkatrienyl group, where the alkyl, alkenyl, alkadienyl and alkatrienyl group may optionally be substituted with 1 or 2 OH groups;

$R_2$ is a straight chain $C_{1-10}$ alkyl group which may optionally be substituted with 1 or 2 OH groups;

with the proviso that the total number p of carbon atoms in $R_1$ and $R_2$ is in the range of $12 \leq p \leq 26$;

or a mixture of such compounds in the clarification and/or deparaffination procedures in the processing of histological tissue samples.

Also, the invention concerns a method for preparing histological tissue samples comprising a clarification step and/or a deparaffination step and in which steps compositions containing the compound of formula I defined above are employed.

Without being bound to any specific theory, it is contemplated that the straight-chain nature of compounds of formula I plays an important role in the function during in particular the clarification phase in that the straight-chain compounds may have a better mobility and therefore be better able to be removed from certain types of tissue than any branched compounds.

DETAILED DESCRIPTION OF THE INVENTION

With respect to the group $R_1$, it is preferred that a straight-chain alkyl group has 7-17, in particular 9-13 carbon atoms, that a straight-chain alkenyl group has 7-17, in particular 9-10 carbon atoms, and that a straight-chain alkadienyl has 7-18 carbon atoms.

In a particularly preferred embodiment of the invention both $R_1$ and $R_2$ are straight-chain alkyl groups.

Specific examples of groups $R_1$ are n-propyl, n-butyl, n-pentyl, n-hexyl, n-heptyl, n-octyl, n-nonyl, n-decyl, n-undecyl, n-dodecyl, n-tridecyl, n-tetradecyl, n-pentadecyl, n-hexadecyl, n-heptadecyl, n-octadecyl, n-nonadecyl, and docecyl, as well as 8-heptadecenyl, 8,11-heptadecedienyl, 8,11,14-heptadecetrienyl and 11-hydroxy-8-heptadecenyl. Thus, for these groups $R_1$, the corresponding acyl group $R_1$-CO- may be an acyl group derived from butyric acid, valeric acid, hexanoic acid, heptanoic acid, caprylic acid, pelargonic acid, capric acid, undecanoic acid, lauric acid, tridecanoic acid, myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid and ricinoleic acid.

Examples of the straight-chain $C_{1-10}$ alkyl group for $R_2$ are methyl, ethyl, n-propyl, n-butyl, n-pentyl, n-hexyl, n-heptyl, n-octyl, n-nonyl, n-decyl. Examples of such groups substituted with 1 or 2 OH groups are 2-hydroxyethyl, 2-hydroxypropyl, 4-hydroxybutyl, 6-hydroxyhexyl. It is preferred that $R_2$ is $C_{1-6}$ alkyl such as methyl, ethyl, n-propyl, n-butyl, n-pentyl or n-hexyl which means that the preferred alcohols from which the esters of formula I are formed are $C_{1-6}$ straight-chain monoalkanols. It is particularly preferred that $R_2$ is $C_{1-4}$alkyl.

A preferred class of esters of formula I are those in which the total number p of carbon atoms in $R_1$ and $R_2$ is in the range $12 \leq p \leq 18$.

Examples of esters of the formula I are n-butyl octanoate, n-butyl decanoate, n-butyl dodecanoate, ethyl dodecanoate, methyl dodecanoate, methyl oleate, methyl linolate, methyl linolenate. Preferred examples that are particularly suitable are n-butyl octanoate, n-butyl decanoate, and n-butyl dodecanoate since these esters combine good solvent properties with good penetration properties, good temperature stability, good stability to hydrolysis, low toxicity, high flashpoint and low odour.

The esters of formula I are either known compounds or may be prepared analogously with known compounds. Thus, a typical process for the preparation of the esters is to react the relevant carboxylic acid with the relevant alcohol in equimolar amounts or with a slight excess of the alcohol in the presence of a catalyst, typically an strong acid catalyst such as sulphuric acid, or, preferably, methanesulphonic acid. The process typically takes place under heating in a reaction vessel which is equipped with means for removing the water formed during the reaction, e.g. a Dean-Stark device or a special separation device depending on whether the alcohol is sufficiently volatile and whether the alcohol is miscible with water or not.

The invention further concerns a method for preparing histological tissue samples, said method comprising one or both of the following steps:

1) a clarification step in which a fixed and partially or fully dehydrated tissue sample is clarified through submersion into a composition A comprising 1-100 % w/w of at least one compound of the formula I defined in claim 1; and

2) a deparaffination step in which a thin section from a tissue sample cast in paraffin is deparaffinated through submersion into a composition B comprising 1-100 % w/w of at least one compound of the formula I defined in claim 1.

As it will be apparent, the compositions used in the clarification and deparaffination steps in the method for preparing histological tissue samples may be pure compounds of the general formula I or mixtures of such compounds or the compositions may be mixtures containing at least one of the compounds of the general formula I in combination with one or more diluents. Thus, it is important that a fluid for clarifying an deparaffination has a low viscosity such as at the most 6 cP ($6 \times 10^{-3}$ kg $m^{-1}$ $s^{-1}$) at the typical operating temperature of 60°C in order to reduce the so-called carry-over whereby the content of one bath will be brought over into the next and thereby polluting it. The present invention fulfils this need in that the esters of formula I have low viscosities which makes them suitable for the clarification and deparaffination processes in histological tissue preparation.

Consequently, it may be desirable to blend the esters of formula I with a diluent in order to decrease the viscosity of the blend to a value lower than the viscosity of the ester of formula I.

Such diluents may be:

1) Saturated hydro-carbon solvents such as odourless kerosene, n-paraffins (saturated n-alkanes), isoparaffins or naphthenes having a flash-point in the range 20-200 °C measured according to ASTM D92;

2) vegetable oils such as coconut oil, palm kernel oil, soy bean oil, rapeseed oil and the like, or processed vegetable oils such as vegetable oils subjected to hydrogenation, refining, bleaching, epoxydation or other conventional oleochemical processes;

3) esters based on $C_{1-24}$ monocarboxylic acids esterified with $C_{1-18}$ mono- or dialcohols not comprised by the general formula I, e.g. 2-ethylhexyl acetate, 2-ethylhexyl 2-ethylhexanoate, 2-ethylhexyl laurate, isobutyl laurate, 2-ethylhexyl oleate and the like;

4) esters based on $C_{2-10}$ dicarboxylic acids and $C_{1-18}$ alcohols, e.g. dimethyl oxalate, dimethyl succinate, dimethyl glutarate, dimethyl adipate; dibutyl phthalate and the like;
5) esters based on mono-, di- or tricarboxylic acids containing one or more hydroxy groups and on $C_{1-18}$ alcohols, e.g. trimethyl citrate, trimethyl acetyl citrate, ethyl lactate and the like;
6) terpenes derived from citrus fruits such as lemons, oranges and limes, an example of such a terpene being d-limonene.

The compositions used according to the invention may contain 1-99 % w/w of the compounds of the general formula I and 99-1 % w/w of the diluents mentioned above, preferably 30-70 % w/w and 70-30 % w/w, respectively, in particular 40-60 % w/w and 60-40 % w/w, respectively.

Other additives that may be be present in the compositions used according to the invention are non-ionic surfactants in an amount of 0.01-10 % w/w, preferably 0.1-5 % w/w, in particular 0.1-0.5 % w/w, e.g. ethoxylated fatty alcohols, ethoxylated castor oil or ethoxylated fatty acids; and $C_{1-10}$ aliphatic alcohols in an amount of 0.01-10 % w/w, preferably 0.1-8 % w/w, in particular 0.1-5 % w/w, e.g. ethanol or propanol.

Generally speaking, compounds of the general formula I or compositions containing such compounds and a diluent may be used as direct replacements for xylene in standardized tissue preparation procedures. Thus, the compounds or the compositions may be used directly in existing tissue processing apparatuses such as the VIP Tissue Processor (Miles, USA), Shandon Elliot Tissue Processor, amd the Autoteknikon Tissue Processor. In a typical process, a tissue sample is first subjected to immersion in a fixing agent such as a 3-4 % aqueous formaldehyde solution followed by immersion in aqueous ethanol solutions of increasing ethanol concentrations going from 70% to 99 %, after which the sample is immersed in the compound of formula I or the composition containing the

compound, usually in several successive but separate baths, in turn followed by immersion in several successive (to ensure removal of the clarifying agent) baths of molten paraffin wax. Usually, the immersions not involving molten paraffin wax are carried out at 40°C whereas the paraffin treatments are usually carried out at 60°C.

In a typical deparaffination procedure, the thin section of the paraffin wax cast is placed on a microscope slide and then placed in a heating chamber at 60°C followed by immersion first into two successive baths of a deparaffinating medium and subsequently into 8 or more baths of ethanol of decreasing concentration at room temperature, typically 4 baths of 99 % ethanol, 2 baths of 96 % aqueous ethanol and one bath of 70 % aqueous ethanol, and finally followed by immersion into water in preparation for staining with conventional staining agents.

When using an ester of the formula I or a composition containing such an ester as the deparaffinating medium, this treatment may be carried out as one treatment at 37°C for 20 minutes followed by a period of 4 minutes at 37°C during which excess deparaffinating medium is allowed to drip off.

The invention is further illustrated by the following preparations, examples and comparative examples.

**PREPARATION 1**

Preparation of n-butyl decanoate

A 1 litre 3-necked flask equipped with a mechanical stirrer, a thermometer, a Dean-Stark water separating device and an inlet for nitrogen was charged with 185 g (2.5 moles) n-butanol, 431 g (2.5 moles) capric acid (n-decanoic acid) of 99% purity, and 3 g methanesulphonic acid. The Dean-Stark device was filled with n-butanol and excess n-butanol (18 g) was

charged into the flask. The temperature was raised gradually to a final reaction temperature of 190°C. After the acid value had fallen to below 0.5 mg KOH/g (tested according to ASTM D974), excess of n-butanol was distilled off in vacuum.

The final product was a thin (viscosity 2 cP at 25 °C), light yellow oil at 20°C with an acid value of approx. 0.5 mg KOH/g.

**EXAMPLE 1**

This example illustrates the use of n-butyl decanoate for clarifying and deparaffinating tissue samples.

n-Butyl decanoate min. 99% purity was applied in a standard Autoteknikon Tissue Processor in the following processing programme for clarification and casting (corresponding to the standard processing programme for the apparatus):

| Programme step no. | Time (min.) | Temp (°C) | Medium |
|---|---|---|---|
| 1 | 60 | 40 | 3.6% formalin |
| 2 | 60 | 40 | 70% ethanol |
| 3 | 60 | 40 | 96% ethanol |
| 4 | 60 | 40 | 96% ethanol |
| 5 | 60 | 40 | 99% ethanol |
| 6 | 60 | 40 | 99% ethanol |
| 7 | 60 | 40 | 99% ethanol |
| 8 | 90 | 40 | n-butyl decanoate |
| 9 | 90 | 40 | n-butyl decanoate |
| 10 | 120 | 60 | paraffin |
| 11 | 120 | 60 | paraffin |

Following casting into paraffin, sections of standard thickness were cut on a microtome and were subsequently put through the following deparaffination procedure:

| Programme step no. | Time (min.) | Temp (°C) | Medium |
|---|---|---|---|
| 1 | 20 | 60 | none (melting max) |
| 2 | 20 | 37 | n-butyl decanoate |
| 3 | 4 | 37 | none (drip-off) |
| 4 | 3 | room | 99% ethanol |
| 5 | 3 | room | 99% ethanol |
| 6 | 3 | room | 99% ethanol |
| 7 | 3 | room | 99% ethanol |
| 8 | 3 | room | 96% ethanol |
| 9 | 3 | room | 96% ethanol |
| 10 | 3 | room | 96% ethanol |
| 11 | 3 | room | 70% ethanol |
| 12 | 3 | room | water |

Subsequent to step 10, 11, or 12 in the above deparaffination procedure, the sections were mounted onto a microscope slide by means of a standard mounting agent (Such as polymethacrylate solution in toluene). Subsequent to step 12, the section was subjected to staining with one of a range of standard staining agents.

A range of different tissue qualities were tested: fatty tissue, non-fatty tissue and blood-rich tissue.

Results: the final tissue preparations were fully comparable with the results obtained when using xylene on the same tissue samples for clarification and deparaffination.

**COMPARATIVE EXAMPLE 1**

A mixture of 60 parts by weight isobutyl octanoate and 40 parts by weight of isobutyl decanoate was tested in a Sheldon Elliot Tissue Processor using the same procedure as described in Example 1. The results showed problems with fatty tissues where the residual concentration of isobutyl octanoate and isobutyl decanoate was too high and caused problems in con-

nection with the cutting of thin sections and in connection with staining.

**COMPARATIVE EXAMPLE 2**

2-ethylhexyl dodecanoate min 99% purity by weight was applied in a Sheldon Elliot Tissue Processor using the same procedure as described in Example 1. The results showed problems with fatty tissues where the residual concentration of 2-ethylhexyl dodecanoate was too high and caused problems in connection with the cutting of thin sections and in connection with staining.

CLAIMS

1. Use of a compound of the general formula I

$$R_1 - C(O) - O - R_2$$

wherein $R_1$ is a straight chain $C_{3-23}$ alkyl group, a straight chain $C_{3-23}$ alkenyl group, a straight chain $C_{5-23}$ alkadienyl group, or a straight chain $C_{7-23}$ alkatrienyl group, where the alkyl, alkenyl, alkadienyl and alkatrienyl group may optionally be substituted with 1 or 2 OH groups;

$R_2$ is a straight chain $C_{1-10}$ alkyl group which may optionally be substituted with 1 or 2 OH groups;

with the proviso that the total number p of carbon atoms in $R_1$ and $R_2$ is in the range of $12 \leq p \leq 26$;

or a mixture of such compounds in the clarification and/or deparaffination procedures in the processing of histological tissue samples.

2. The use according to claim 1 wherein

$R_1$ is a straight chain $C_{7-17}$, preferably $C_{9-13}$, alkyl group, a straight chain $C_{7-17}$, preferably $C_{9-10}$, alkenyl group, a straight chain $C_{7-18}$ alkadienyl group or a $C_{7-18}$ alkatrienyl group, where the alkyl, alkenyl, alkadienyl and alkatrienyl group may optionally be substituted with 1 or 2 OH groups.

3. The use according to claim 1 or 2 wherein $R_2$ is a $C_{1-6}$ alkyl group, preferably a $C_{1-4}$ alkyl group.

4. The use according to any of claims 1-3 wherein p is in the range $12 \leq p \leq 18$.

5. The use according to any of claims 1-4 wherein both $R_1$ and $R_2$ are straight chain alkyl groups.

6. The use according to any of claims 1-5 wherein the compound of formula I is n-butyl decanoate, n-butyl dodecanoate.

7. A method for preparing histological tissue samples, said method comprising one or both of the following steps:

1) a clarification step in which a fixed and partially or fully dehydrated tissue sample is clarified through submersion into a composition A comprising 1-100 % w/w of at least one compound of the formula I defined in claim 1; and

2) a deparaffination step in which a thin section from a tissue sample cast in paraffin is deparaffinated through submersion into a composition B comprising 1-100 % w/w of at least one compound of the formula I defined in claim 1.

8. A method according to claim 7 wherein compositions A and B further comprise one or more diluents selected from

1) saturated hydrocarbon solvents such as odourless kerosene, n-paraffins (saturated n-alkanes), isoparaffins or naphthenes having a flash-point in the range 20-200 °C measured according to ASTM D92;

2) vegetable oils such as coconut oil, palm kernel oil, soy bean oil, rapeseed oil and the like, or processed vegetable oils such as vegetable oils subjected to hydrogenation, refining, bleaching, epoxydation or other conventional oleochemical processes;

3) esters based on $C_{1-24}$ monocarboxylic acids esterified with $C_{1-18}$ mono- or dialcohols not comprised by the general formula I, e.g. 2-ethylhexyl acetate, 2-ethylhexyl 2-ethylhexa-

noate, 2-ethylhexyl laurate, isobutyl laurate, 2-ethylhexyl oleate and the like;

4) esters based on $C_{2-10}$ dicarboxylic acids and $C_{1-18}$ alcohols, e.g. dimethyl oxalate, dimethyl succinate, dimethyl glutarate, dimethyl adipate; dibutyl phthalate and the like;

5) esters based on mono-, di- or tricarboxylic acids containing one or more hydroxy groups and on $C_{1-18}$ alcohols, e.g. trimethyl citrate, trimethyl acetyl citrate, ethyl lactate and the like;

6) terpenes derived from citrus fruits such as lemons, oranges and limes, an example of such a terpene being d-limonene.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00477

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 1/28, G01N 1/34
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | EP, A1, 0010750 (HOECHST AKTIENGESELLSCHAFT), 14 May 1980 (14.05.80), page 8, line 31 - page 9, line 3 | 1,7 |
| A | DE, A1, 3400195 (CARL ROTH GMBH & CO), 11 July 1985 (11.07.85) | 1,7 |

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

09/02/95

International application No.
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| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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